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In the second year, a HTc coil has been fabricated and tested. The resonance frequency is within the target frequency range of the original design. The Q value for the coil is 650 and it is expected to increase when the coil is operated below 40 ^{0}K. The metabolic and cell proliferation differences of MCF7 wild type cells and its multidrug resistant variants were studied. Some subtle difference in the spectra from these two cell lines can not be confirmed. This is subtle difference may be detectable with the high sensitivity HTc probe. The drug sensitive MCF7 cells were dramatically affected by 2 μM Doxorubicin within two hours perfusion and not responsive to Tamoxifen up to 12 hours. In contrast, MCF7/ADR multidrug resistant cells show no effect by perfusion of 2 μM Doxorubicin.

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I. INTRODUCTION

Conventional mammography has been shown to play an important role in detection and staging of breast cancer in older women. For younger women who frequently have radiodense breast tissue or women with silicon implants, rendering breast cancer diagnosis with conventional mammography is problematic. When mammographic findings and clinical findings concur regarding the possibility of a lesion being malignant, usually a fine-needle aspiration biopsy will be performed for definitive diagnosis. The false positive rate is high; only 20-30% of lesions suspicious for cancer at mammogram is actually positive for cancer at biopsy. In general, mammography is limited to detect a tumor several millimeters or larger in size. Because of difficulty with early detection, clinicians are sometimes limited to treat larger size cancers, which in many cases have already metastasized. Accurate definition of tumor size, number, and margins is highly critical in the clinical determination of conservation treatment versus mastectomy. A role exists for an imaging method that can improve sensitivity for detection of small lesion and to improve the specificity for better staging of the disease. To provide the best chance of overall survival, breast cancers need to be accurately staged for systemic treatment and optimal conservation surgery. Traditionally, the gold standards for such assessments are clinicopathological staging and histopathological typing and grading of malignancy. In the classical histopathological approach problems exist inherently, predominantly, the accuracy of the initial biopsy procedure and the variable skills applied to its histological assessment. Development of a new modality to remove sampling errors, improve specificity and produce a grading of tissues that relates to establish biological criteria would be very useful. Over the last few years, magnetic resonance imaging (MRI) and spectroscopy (MRS) have emerged as one of the most promising clinical tools to fill the gap between clinical needs and information obtained by conventional breast imaging and pathological methods. Preliminary results indicate that MRI may be more sensitive than conventional x-ray mammography in detecting small lesions. Cancers have typical metabolic characteristics in ^{31}P and ^{1}H MRS including high levels of phospholipid metabolites and a cellular pH more alkaline than normal. Although these alone are not unique for cancer but they are very useful diagnostic information in appropriate clinical settings. MRS is capable of distinguishing benign and malignant lesions in a particular anatomical site and to be a specific diagnostic discriminant in a particular situation. It has been demonstrated to be useful to improve the specificity of the MR imaging of breast. Some metabolic characteristics appear to be prognostic indices and correlate well with the response of treatment. The improvement of specificity will reduce the number of biopsies performed to confirm false-positive mammographic findings and more effectively to assess the results of treatment. Many of these progresses are based on the advances of NMR studies of the perfused breast cancer cells and tumor-bearing animal models. One of the major limitations of the application of NMR methods both in vitro and in vivo is its low sensitivity. The sensitivity determines the ultimate cancer detection capability and the resolution in image and in spectrum. In this study, a high temperature superconductor working at very low temperature will be used to reduce electronic noise and significantly improve the sensitivity of detection. It will dramatically increase the sensitivity and improve the resolutions. The improvement will be verified by comparison the sensitivity

with a conventional probe. The improvement of detection sensitivity will provide a more accurate diagnosis and it may become possible for early prediction of tumor response to therapy. The probes will be constructed with $YBa_2Cu_3O_7$ material and to be tested on two well defined experiments: an in vivo cell metabolism study on a 9.4 T spectrometer and an in vivo tumor bearing animal study on a 4.7 scanner. In the cell metabolism study, breast cancer cell line MCF7 and its variants will be studied in terms of characteristic differences of their ^{31}P spectra during growth phase and under effects of Tamoxifin. In the in vivo animal study, MCF7 cells and its variants will be grown as xenografts on nude mice. The differences of ^{31}P spectra during progress of tumor and responses to Doxorubin and Tamoxifen will be studied. The high resolution proton imaging experiments of vasculature of tumor will be conducted using both conventional copper probe and the proposed HTS probes.

In the first year of the project, the design of self-resonant probes for high-resolution nuclear magnetic resonance (NMR) has been completed. The receiver coil uses thin-film, high temperature superconductor, $YBa_2Cu_3O_7$.The transmitter coil is a standard room-temperature coil. The probe is designed to fit either a 9.4 T machine for in vivo cell study or a 4.7 T machine for animal study. The coils are detachable so that different coils can be substituted in and out of the different machines and for different nuclei. Three identical cell perfusion apparatus for the NMR study of breast cancer cell metabolism have been constructed and tested. The apparatus was tested using known standard compounds. To study the metabolism of breast cancer cells in an extended period of time, the cells are continuously perfused with nutrients. During perfusion, the breast cancer cells are restrained in the agarose gel-thread matrices. A protocol for making agarose gel-thread matrices containing MCF7 breast cancer cells is established. Besides the above-mentioned tasks, some of the infrastructure and preparation works necessary for conducting the proposed research have been accomplished. The 400 MHz machine has moved to the new laboratory next to the other NMR machines. This improves the logistics of the research. Two new laboratories have been renovated to accommodate the NMR machines and cell biology work. Much equipment and computer hardware and software have been ordered and installed. Major software upgrade has been done on the 200 MHz machine. A diffusion imaging technique has been implemented on the 200 MHz machine. It is ready for the in vivo micro-imaging study of tumor vasculature and drug delivery inside the tumor.

The main tasks for the second year are: fabrication of the HTc coils, integration of the probe, and ^{31}P studies of spectroscopic differences and responses to Tamoxifen and Doxorubicin of MCF7 cells and its variants. In order to perform the in vivo cell study, some systematic check such as RF calibration, magnetic field drift, and cell culture contamination are conducted first.

II. BODY

II.1 High Tc Probe

Fabrication and Measurement of High Tc RF Coils

The 400 MHz coil was fabricated by depositing a thin film of the high Tc superconducting material ($YBa_2Cu_3O_7$) onto a 1 x 1 cm lanthanum aluminate ($LaAlO_3$) substrate (Figure 1). This thin film was lithographically patterned to define the coil and the interdigitated capacitor, and the unwanted material was removed with an ion mill etch. The coil has been protected from the environment by applying a thin layer of Teflon to cover the superconducting thin film. Our target resonance frequency is 392 MHz. We measured the resonance frequency of this coil using a small loop of copper wire to magnetically couple to it. The coil was immersed in liquid nitrogen and the impedance was measured by the copper loop. The measured resonance frequency at 77 ^{0}K was 401.6 MHz. Further cooling to the operating temperature of the cryostat should reduce the resonance frequency very slightly, and it will remain about 2.5 % above the target value. This is better than we expected for our first design. The Q of the coil as measured in this way was only 650. It is not as high as desired because for the measurement was performed at 77 ^{0}K and the coil was mounted on a piece of composite PC board material. When the coil is mounted in the cryostat on a sapphire substrate and cooled below 40 ^{0}K, the Q should increase dramatically.

The high Tc superconducting coils have been designed to have an untuned resonant frequency slightly below the required final resonance frequency. The size of the coil is selected to match the active sample length and the width is optimized for coupling. The inductance between the high Tc superconductor the interdigitated capacitor was designed to give a sufficient adjustable frequency. The coil is designed to have a target resonance frequency 2 % below the final frequency, which allows the range to trim out process variations. This initial trim consists of a lithography and etching step. After the initial trim, it moves the resonance frequency close to within 0.5 % of the target frequency. The final tuning will be accomplished inside the magnet by the tuning mechanism designed into the cryostat. In order to compensate for the variation between samples, a metallic tuning paddle is added to adjust the resonance frequency through inductive coupling. The tuning range of such a design can be as large as a few MHz. It is to accommodate the range resonance frequency shift from different samples.

Integration of the Probe

The probe consists of an Oxford Spectrostat cryostat with some custom-machined parts attached to the cold head. The custom cold head space contains the mounting facilities for the high Tc RF coil, the fine tuning paddle and a copper impedance matching loop to the preamp. The whole system is under assembly and testing at Quantum Magnetics. The same probe will be used for both in 400 MHz vertical bore as well as in the 200 MHz horizontal bore machine with a slightly rearranged cold head. The rearrangement consists of changing the external form of the cold head from one, which accepts a test tube (for the 400 MHz vertical system), to a one-sided flat surface (for the

200 MHz horizontal system). No other changes of the mounting the coil, tuning or the readout mechanism are needed.

II.2 System Preparation for In Vivo Cell Study

RF pulse measurement and magnetic field drift test

In order to achieve the best signal-to-noise (S/N) ratio within a fixed period of data acquisition time, the RF pulse width and the transmission power need to be optimized. The optimal RF depends on the repetition time and the T_1 relaxation time of the sample. The T_1 relaxation time of the inorganic phosphate was used for calibration of the RF power. The inversion recovery experiment was used to measure T_1 of inorganic phosphate in the perfusion medium. The measured T_1 value of inorganic phosphate was 5.4±0.9 seconds. Based on this information the initial parameters of the NMR experiments were determined.

Another system quality control process, viz., determining the effect of magnetic field drift, was established also. Since our perfusion experiment requires long data acquisition time, the effect of magnetic field drift may increase the line width of ^{31}P signal and reduces the resolution. A 10% trimethyl phosphine (TMP) in C_6D_6 was used to study the magnetic field drift. Figure 2 shows a spectrum obtained with 1000 scans and the total scan time was 5800 seconds. The line width of each individual peak is about 5.2 to 5.8 Hz. When eight such spectra were accumulated consecutively and added together, the result of such summation shows a spectrum with broader line width and lower resolution (Figure 3). The eight distinct peaks were blurred in Figure 3. This is due to the effect of small magnetic field drift. Comparing the three highest peaks in the spectrum, the frequency shift was about 5 Hz. It corresponds to a shift of 0.031 ppm during the total acquisition time of 13 hours. In a perfused cancer cells study, the closest peaks are at least 0.5 ppm apart. The effect of field drift is negligible. In fact, the quality of shimming and the line broadening introduced routinely in the data processing of 20 to 30 Hz will cause a much bigger effect on the spectrum than the magnetic field drift. Therefore, the field drift effect of the spectrum is negligible.

Upgrade the incubator and certify the tissue culture hood

In the past during the incubation, HEPES was added to the growth media in order to maintain a constant pH due to poor control of CO_2 supply in our old incubator. At the beginning of this year, we have installed a new incubator with adequate control of CO_2. It makes the use of HEPES in the media unnecessary. The sterilized hood for tissue culture work is certified annually. A strict disinfecting procedure is imposed. The hood is restricted to our study to avoid potential contamination from other experiments. HEPES, however is still used in the medium in the NMR experiment.

Verification of potential cell contamination

We have experienced some problems with cell contamination at the beginning of in vivo study. The observed abnormalities of cell culture include slow cell growth rate, patches of void area in the monolayer, easy detachment with trypsin and cell death. A Mycoplasm test (GIBCO Cat. No. 15672-017) had been conducted and it failed to detect any trace of mycoplasm contamination. In order to find out what are the factors affecting the growth in our culture, a series of detailed tests have been performed. To identify the source of contamination is a complicated problem. The contamination may due to multiple factors. The tests include using different growth media with or without phosphate, serum from different vendors, fungicide (amphotericin B), gentamicin, penicillin G and streptomcin, presence of HEPES in the growth media. Several media with and without HEPES are used to test if the cells were subject to pH stress. It is an indirect test of a proper CO_2 level in the incubator. We used media with or without fungicide and/or antibiotics, to test whether cells were infected, if it is infected, what kind of infection it was. The results from these tests indicate that some of our cell lines were indeed infected based on drastic difference in spectra from antibiotics containing and antibiotics free media. Consequently, new cell lines from NCI were obtained and used in the later experiments. We also found that in some studies the cells in the exponential growth phase did not show any difference in antibiotics containing or antibiotics free media. This suggests other factors may affect the cell culture. This leads to a study of comparing effects of serum from different vendors and investigating the possibilities of other components in the media such as concentration of phosphate and HEPES. We did find significant difference with serum from different vendors. Sometimes, even from the same vendor but different batch shows different results.

II.3 ^{31}P Spectra of MCF7 Cells and Its Variants

MCF7 breast cancer cells were grown as conventional monolayers and harvested as single cell suspensions, which were then embedded in agarose gel threads. These cells embedded in agarose threads were transferred to a NMR tube and then perfused with culture medium. The culture medium contains IMEM supplemented with 10% heat inactivated fetal bovine serum, glutamine (2 mM), sodium pyruvate (1 mM) and the antibiotic penicillin and streptomycin. The details of cell culture and making agarose gel-thread matrices were reported last year. Using the cell perfusion apparatus, we conducted a series of ^{31}P studies of the wild type MCF7 cells (Figure 4) as well as the drug resistant MCF7/ADR cells (Figure 7). Many phosphorus metabolites can be identified such as: phosphocholine (PC, 3.75 ppm); inorganic phosphate (Pi, 1.5 ~ 2.67 ppm); γ-ATP (-4.98 ppm); α-ATP (-10.06 ppm); β-ATP (-18.59 ppm); diphosphodiesters (DPDE, -10.7 plus –12.41 ppm), glycerophosphoethanolamine (GPE, 0.98 ppm) and glycerophospho-choline (GPC, 0.45 ppm). In the Figure 4, the number of transient is 4000. The repetition time is 1.1 seconds. The line broadening is 20 Hz. We noticed the GPC and GPE peaks are influenced by the minor differences in the media and the cell density.

Since the signal-to-noise ratio of the NMR spectrum is usually low, sometimes a long scan 12 hours or longer is needed. It is important to verify the viability of the cells

during a long period of time. Figure 5 depicts the changes observed in the intensities of the different phosphorus metabolites over a fourteen-hour period. The observed constant amount of ATP (based on ATP-beta signal) indicates that the cells are still viable after 12 hours perfusion. The failure to observe increasing amounts of ATP indicates that the cells are not proliferating when embedded in agarose threads. We have initiated experiments using matrigel for embedding the cells. The cells are expected to grow on a matrigel matrix perfused with complete growth medium.

II.4 Effects of chemotherapy drugs and growth effectors

We have studied the differences in the ^{31}P NMR spectroscopic profile for drug sensitive MCF7 cancer cells and their multidrug resistant variant MCF7/ADR cells. We have access to several variants of MCF7 cells, but have chosen to investigate MCF7/ADR cells first because we have already studied the growth characteristics and drug response of these cells. Figure 6 shows a significant difference on the proliferation of these two cell lines under the influence of glucose. However, the multidrug resistant MCF7/ADR cells did show glucose dependent to the cell proliferation. These cell lines were grown as conventional monolayers and harvested as single cell suspensions, which were then embedded in agarose gel threads. Same as previous experiments, these cells embedded in agarose threads were transferred to a NMR tube and then perfused with culture medium with or without chemotherapeutic drugs: tamoxifen and doxorubicin. The effects of these drugs on the ^{31}P NMR spectra were evaluated in order to highlight the differences between the drug sensitive and drug resistant cell lines. Differences between the two cell lines were clearly seen.

When drug sensitive MCF7 wild type cells were embedded in agarose and perfused with medium first without tamoxifen for one hour then switched to tamoxifen containing medium (8 microgram/ml). No significant changes were observed (Figure 7). When these cells were perfused with medium containing 2 μM doxorubincin for 10 hours, a significant decrease in the ATP was observed along with an increase in the intensity of the inorganic phosphate signal (Figure 8). However, when multidrug resistant MCF7/ADR cells embedded in agarose threads were perfused with 2 μM doxorubicin there was no change in ATP (Figure 9). There are sufficient fine structure differences in the spectra obtained for the drug sensitive MCF7 cells and the multidrug resistant MCF/ADR cells. The difference is currently under investigation. It will be interesting to study and document these differences using the high temperature probe which is expected to increase the signal to noise ratio.

III. CONCLUSIONS

In the second year of this project, we have fabricated and tested a HTc coil, as well as studied the ^{31}P spectroscopic differences of MCF 7 cells and its variants and their responses to Tamoxifen and Doxorubicin. Based on the test done at 77 ^{0}K, the HTc coil has a resonance frequency 401.6 MHz and the Q value for the coil is 650. This is better

than we expected in the design. Since the coil is going to be mounted in the cryostat on a sapphire substrate and cooled below 40 ^{0}K, the Q should increase further. The integration of the probe is prolonged due to the delay of the delivery of cryostat from Oxford Instrument. The whole system is under ensemble and to be tested at Quantum Magnetics (the subcontractor of the project). We have studied the differences in the ^{31}P NMR spectroscopic profiles for drug sensitive MCF7 cancer cells and their multidrug resistant variant MCF7/ADR cells using a conventional probe. The cells are embedded in agarose gel threads and perfused with growth medium during the NMR studies. Many detailed phosphorus metabolites can be identified. There may be some subtle differences in the spectra of the two cell lines. However, the differences are not conclusive using the conventional probe. We have successfully demonstrated drug sensitive MCF7 cells which were dramatically affected by 2 μM Doxorubicin within two hours perfusion and not responsive to Tamoxifen up to 12 hours. In contrast, 2 μM Doxorubicin was without any effect on multidrug resistant MCF7/ADR cells and the ^{31}P NMR spectrum did not differ appreciably after addition of Doxorubicin. In order to have a highest S/N in the in vivo studies, a great deal of efforts have been made to ensure a reliable NMR system and a contamination free cell culture environment. The scan parameters are optimized. The magnetic field drift during the long acquisition time is negligible. All the potential cell contamination sources are eliminated.

In summary, the HTc coil is constructed and tested. However, the final probe is still in the process of being assembled at the Quantum Magnetics. A great deal of progress has been made in cell metabolism study including obtaining ^{31}P spectra from MCF7 wild type cells and its variants. The MCF7 proliferation study is in progress. The study of the effects of Tamoxifen and growth factors on these breast cancer cell lines is almost complete.

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APPENDICES

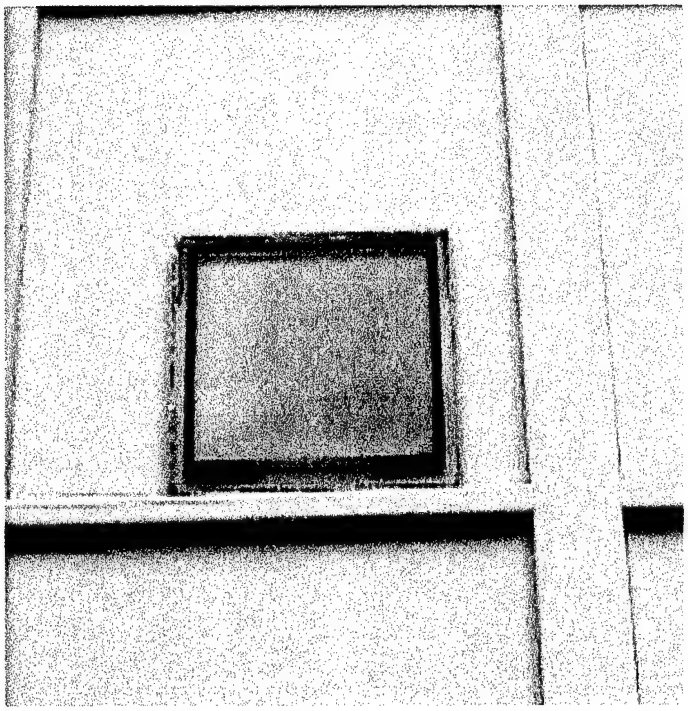

Figure1: Photograph of the prototype 400 MHz high Tc superconducting resonant coil. The substrate is 1 x 1cm $LaAlO_3$, the black material just within the edge of the substrate is the $YBa_2Cu_3O_7$ film, and the lighter gray area in the middle of the bottom trace is the interdigitated capacitor.

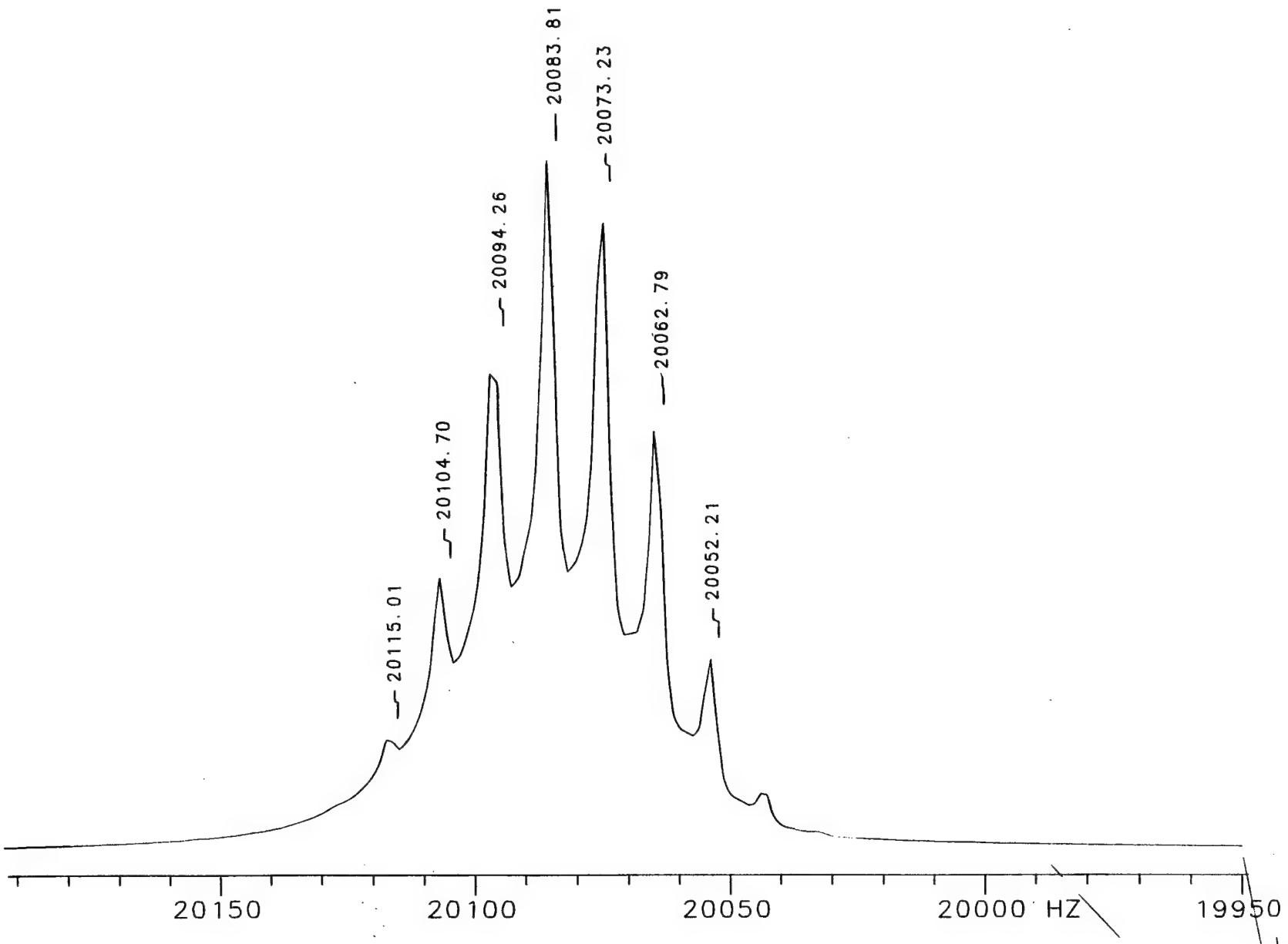

Figure 2. A ^{31}P spectrum of 10% trimethyl phosphine (TMP) in C_6D_6. The number of transients is 1000. Total scan time is 5800 seconds. All eight peaks are well resolved. The line width of individual peaks is about 5.2-5.8 Hz.

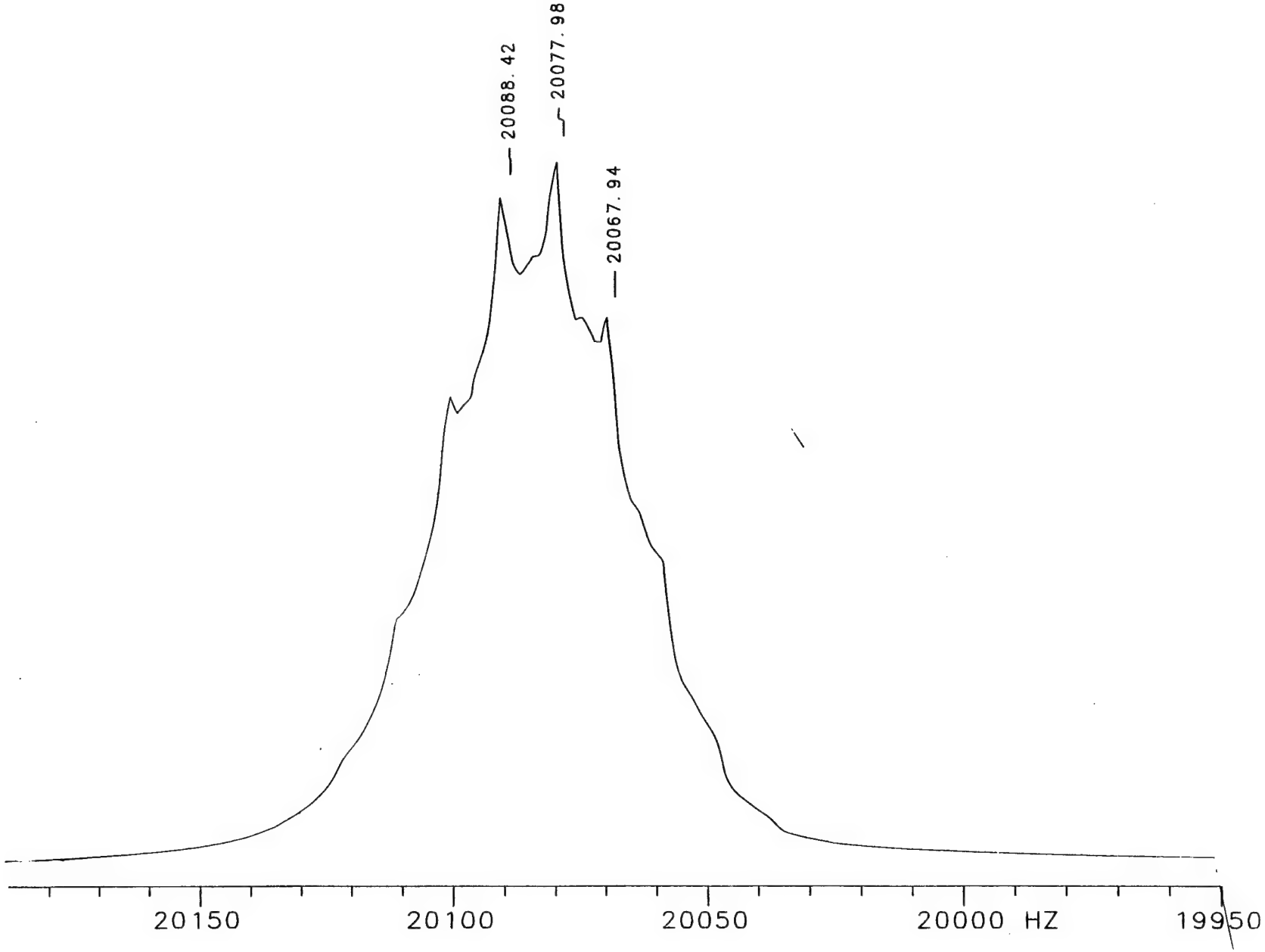

Figure 3. The accumulation of eight spectra in Figure 2 over 13 hours. Significant line broadening is due to the magnetic field drift during this period of time. The frequency shift is about 5 Hz (or 0.031 ppm). This is negligible in our in vivo cell studies because the closest peaks are at least 0.5 ppm apart.

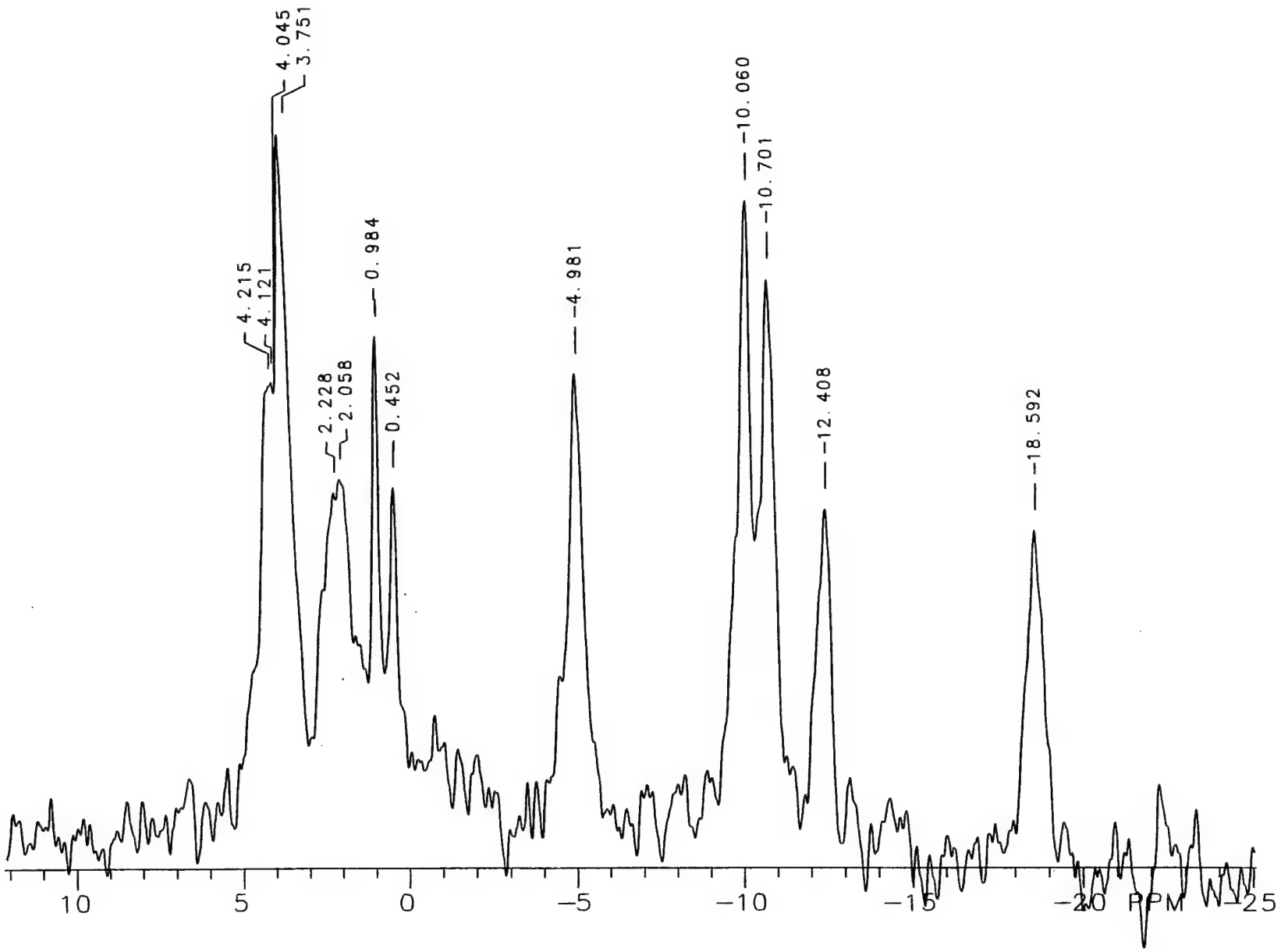

Figure 4. A ^{31}P spectrum of MCF7 wild type cells. The cells are embedded in agarose gel threads perfused with complete growth medium. Many phosphorus metabolites can be identified such as: phosphocholine (PC, 3.75 ppm); inorganic phosphate (Pi, 1.5 ~ 2.67 ppm); γ-ATP (-4.98 ppm); α-ATP (-10.06 ppm); β-ATP (-18.59 ppm); diphosphodiesters (DPDE, -10.7 plus –12.41 ppm), glycerophosphoethanolamine (GPE, 0.98 ppm) and glycerophospho-choline (GPC, 0.45 ppm).

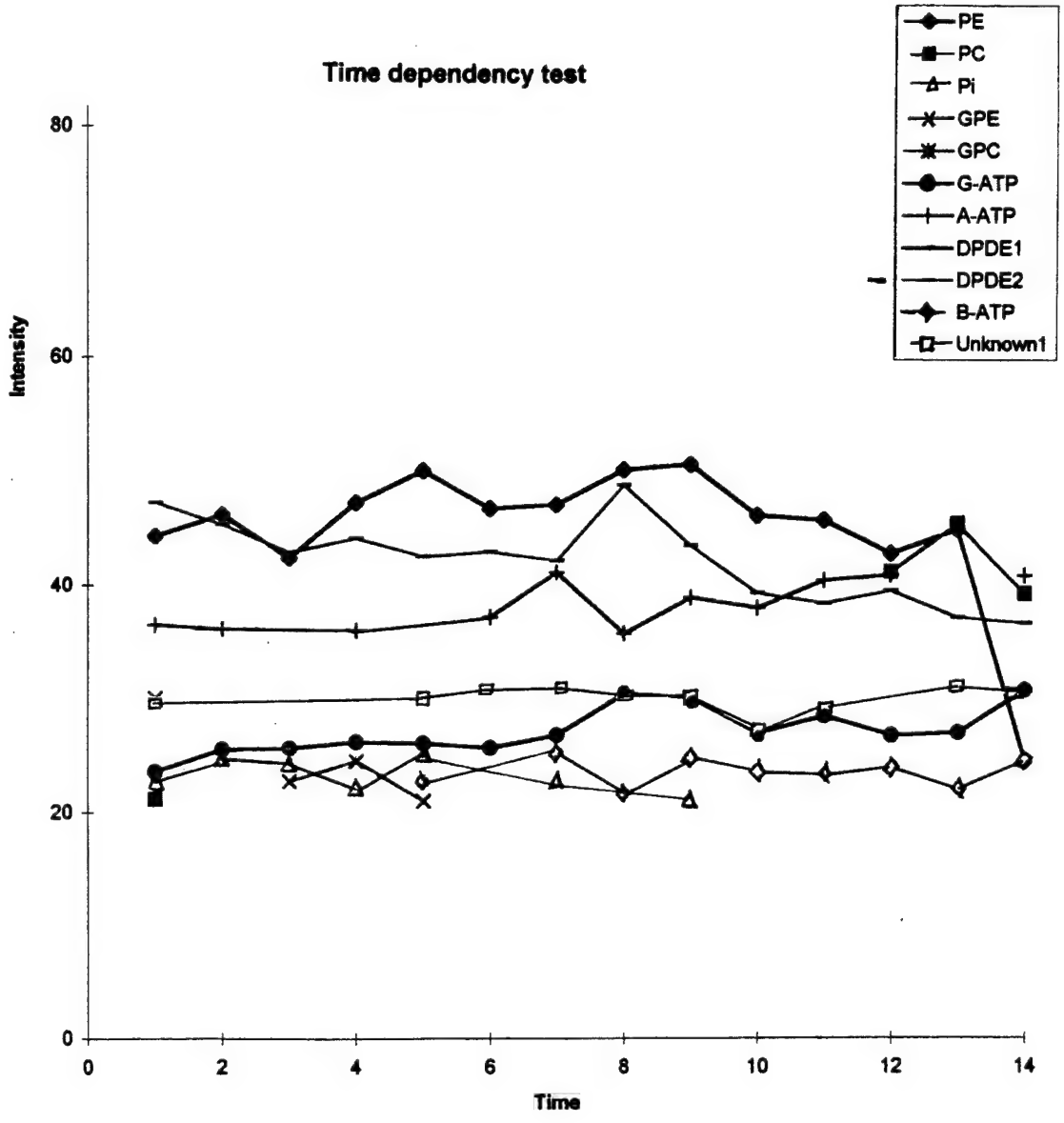

Figure 5. Concentrations of phosphorus metabolites in time from MCF7 wild type. The MCF7 cells are in agarose gel and perfused with growth medium. Over fourteen hours period, all the concentrations of the metabolites are maintained constant.

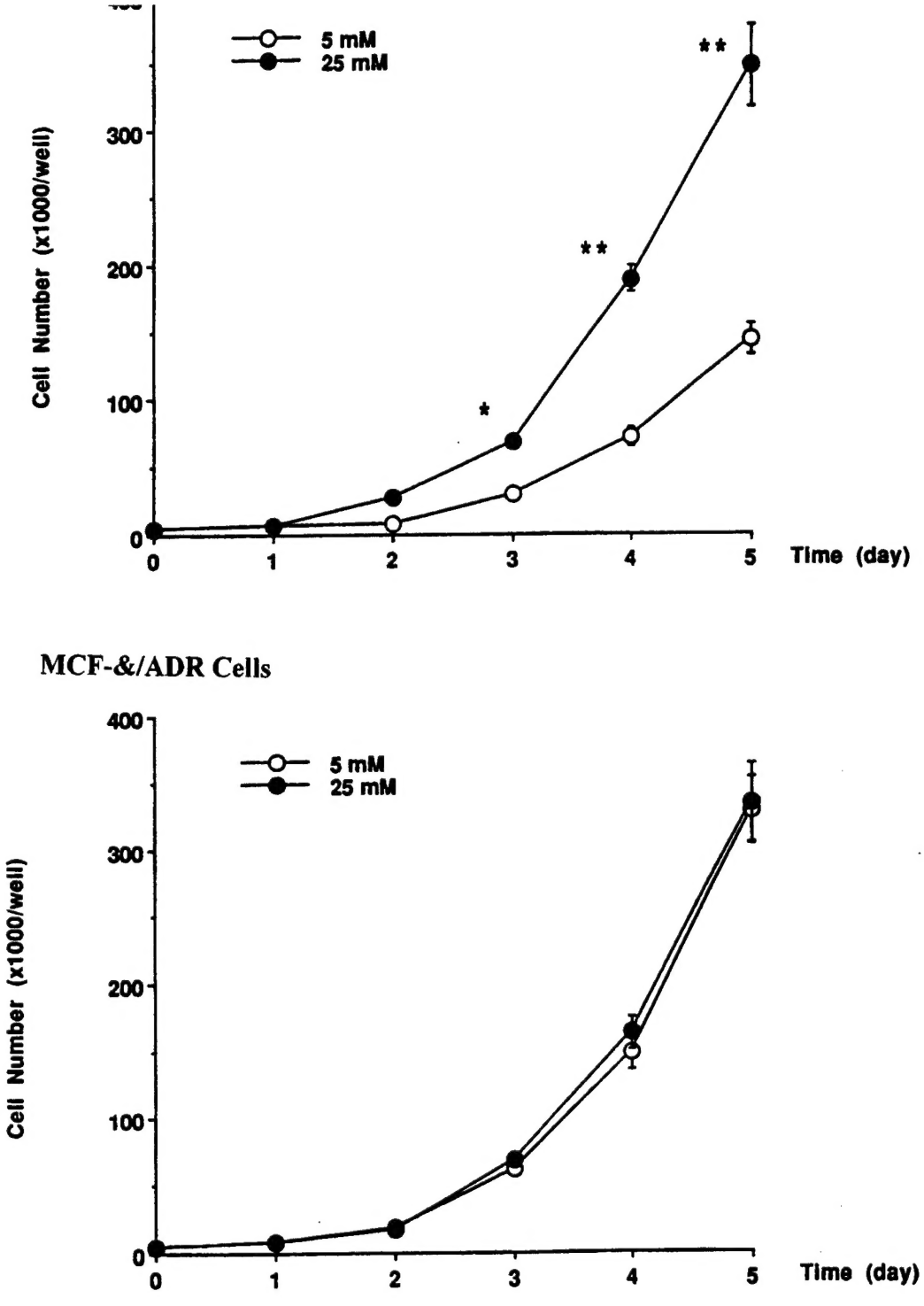

Figure 6. The effect of glucose concentration in culture media. (a) The proliferation of MCF7 wild type cells shows a glucose dependent relationship. (b) For MCF/ADR multidrug resistant cells, it shows independent of glucose concentration.

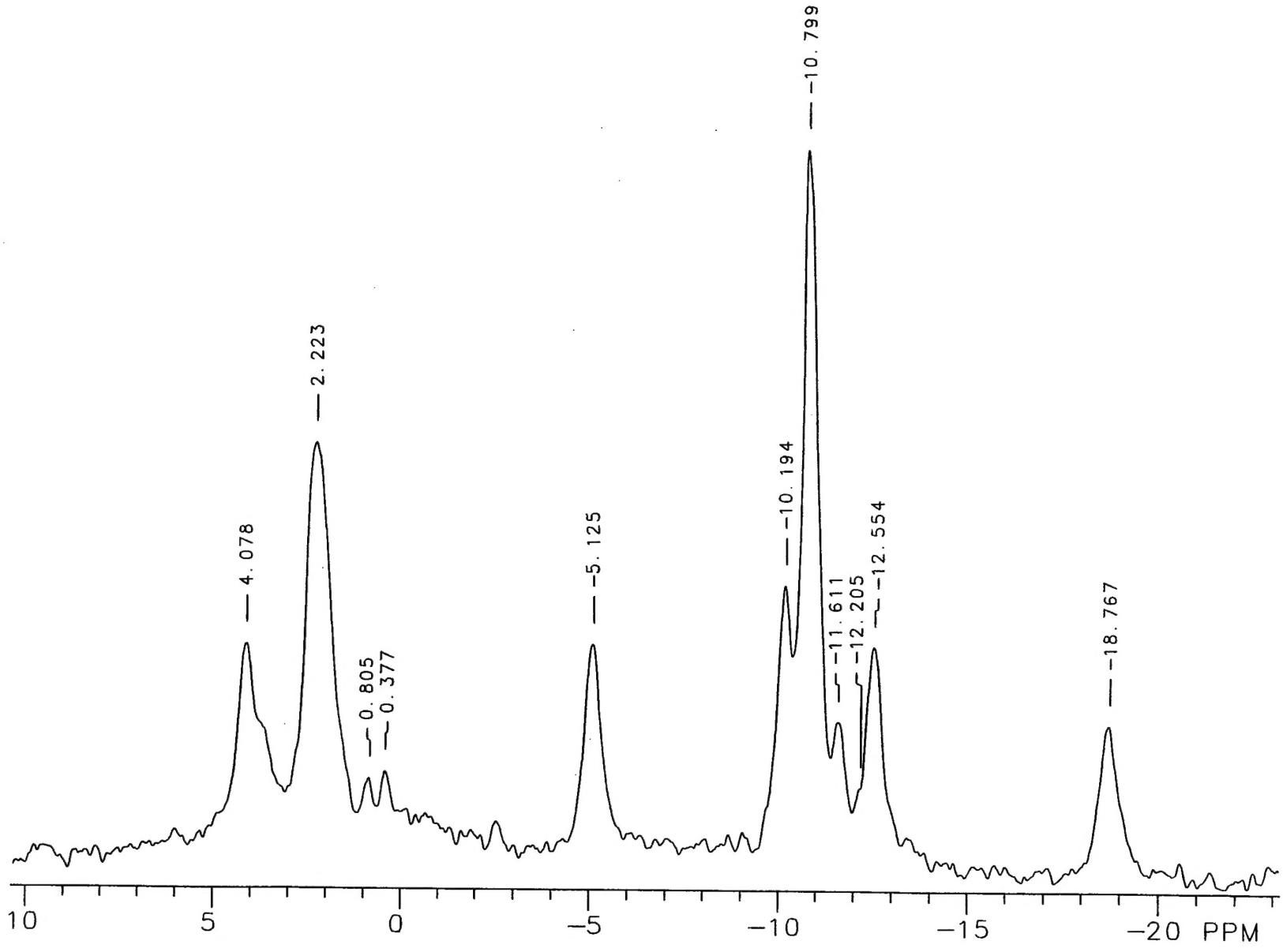

Figure 7. A ^{31}P spectrum of drug sensitive MCF7 wild type cells treated with Tamoxifen (8 μg/ml). The repetition time is 1 second. The number of transient is 2000. The RF pulse width is 36.1 μs. There is no difference compared with a spectrum taken from a Tamoxifen free medium.

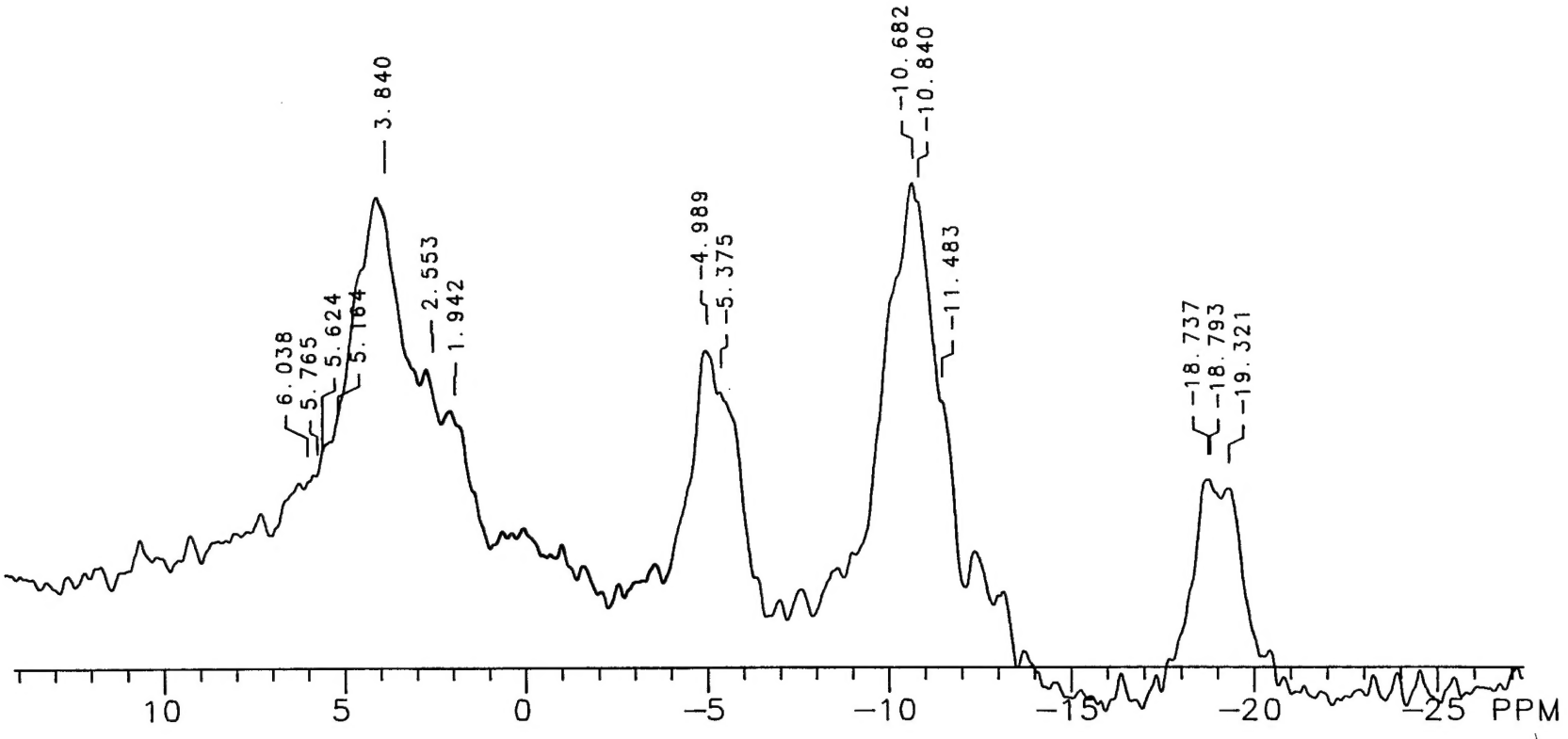

Figure 8. A ^{31}P spectrum of drug sensitive MCF7 wild type cells treated with 2 μM doxorubicin for 10 hours. The repetition time is 1 second. The number of transient is 2400. The RF pulse width 55 μs. The repetition time is 1 second. The spectrum is an accumulation of 10 hours. The ATP peaks are down and the Pi peak increases.

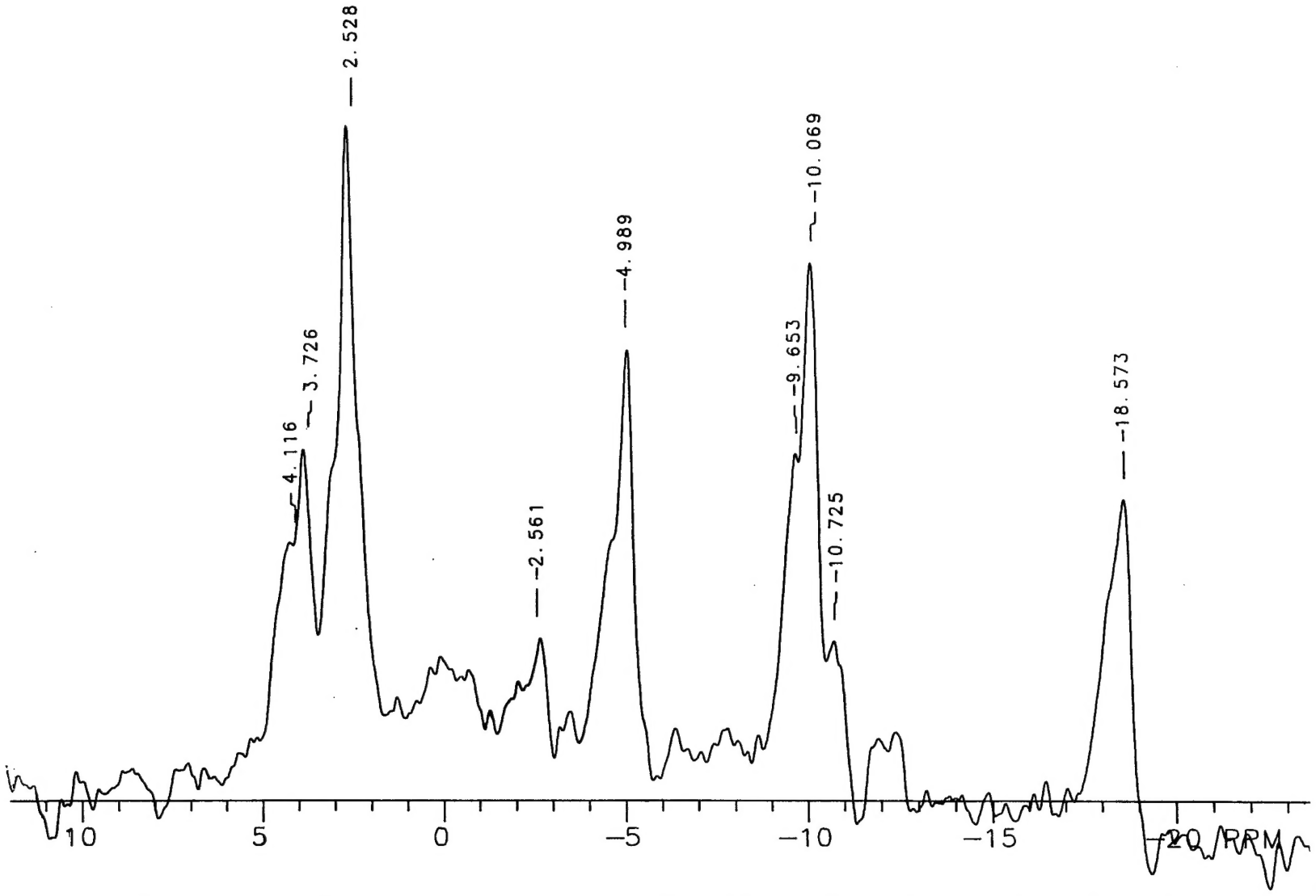

Figure 9. A ^{31}P spectrum of drug resistant MCF7/ADR cells. The perfusion medium contains 2 μM doxorubicin. There is no change in ATP compared to the spectrum taken before doxorubicin was introduced. The total scan time was 12 hours. All the scan parameters are the same as in Figure 8.